(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
30 January 2003 (30.01.2003)

PCT

(10) International Publication Number
WO 03/008632 A1

(51) International Patent Classification[7]: C12Q 1/00

(21) International Application Number: PCT/SE02/01391

(22) International Filing Date: 16 July 2002 (16.07.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
0102549-3 16 July 2001 (16.07.2001) SE
60/305,159 16 July 2001 (16.07.2001) US

(71) Applicants and
(72) Inventors: **HÖGESTÄTT, Edward** [SE/SE]; Kyrkoled 15, S-227 31 Lund (SE). **ZYGMUNT, Peter** [SE/SE]; Måsvägen 16 B, S-227 33 Lund (SE).

(74) Agent: **STRÖM & GULLIKSSON IP AB**; Sjöporten 4, S-417 64 Göteborg (SE).

(81) Designated States *(national)*: AE, AG, AL, AM, AT (utility model), AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ (utility model), CZ, DE (utility model), DE, DK (utility model), DK, DM, DZ, EC, EE (utility model), EE, ES, FI (utility model), FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK (utility model), SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 03/008632 A1

(54) Title: FATTY ACID CONJUGATION AS A METHOD FOR SCREENING OF POTENTIALLY BIOACTIVE SUBSTANCES

(57) Abstract: The present invention relates to a method for screening of compounds or molecules that undergo conjugation with fatty acids present in a mammal body, including humans, whereby a compound to be tested is brought into contact with a fatty acid together with a tissue homogenate or an enzyme, whereupon any conjugation between the compound and any fatty acid present in said homogenate is determined with regard to presence of fatty acid amides, esters or thioesters, as well as a kit for conjugation of such compounds or molecules with fatty acids.

TITLE

FATTY ACID CONJUGATION AS A METHOD FOR SCREENING OF POTENTIALLY BIOACTIVE SUBSTANCES

DESCRIPTION

Field of invention

Method for screening of compounds or molecules that undergo conjugation with fatty acids present in a mammal body, including humans, as well as a kit for fatty acid conjugation of such compounds.

Background of invention

High throughput screening (HTS) of compound libraries allows the testing of large numbers of substances for biological activity. Despite this novel strategy for drug discovery, the number of "hits" remains relatively low. Due to problems related to bioavailability, toxicity and drug synthesis, most drug discovery programs in the pharmaceutical industry focus on compounds with low molecular weight. It is also known that substances may undergo extensive metabolic transformation *in vivo* to bioactive molecules. Such enzymatic bioactivation may involve oxidation, hydrolysis and conjugation of the parent molecule, or combinations of these reactions. It is therefore possible that many compounds showing no or little activity in HTS assays and therefore discarded would have been effective when given to whole animals due to metabolic conversion to bioactive molecules. Methods to predict such biotransformation and to systematically synthesize potential bioactive drug metabolites would be of great value to increase the number of hits in HTS assays.

Summary of the invention

Paracetamol (acetaminophenol) is a frequently used antipyretic and analgesic agent, which differs from most non-steroidal anti-inflammatory drugs (NSAIDs) in that it is a weak anti-inflammatory agent and does not produce the typical side effects related to cyclo-oxygenase (COX) inhibition (7, 15). Although acetaminophen was introduced into clinical medicine more than a century ago, its mechanism of action is still unknown. A selective inhibition of prostaglandin synthesis in brain, consistent with a central site of action of acetaminophen (3, 18), has been proposed (12). However, acetaminophen is a very weak inhibitor of isolated COX (Fig. 1), and there are now clear indications that the analgesic effects of acetaminophen involve molecular targets distinct from COX (1, 14, 20).

Much interest has been focused on vanilloid (VR1) and cannabinoid (CB1 and CB2) receptors as drug targets for treatment of pain and inflammation (22, 23). Both vanilloid and cannabinoid receptors are present in the pain and thermoregulatory pathways and mediate analgesia and hypothermia, and they also display an overlap in ligand recognition properties (21-24). Others and we have recently reported that the fatty acid amide AM404 is a potent activator of rat (Fig. 2) and human vanilloid receptors (21, 27). AM404 is also a ligand at cannabinoid receptors and an inhibitor of the anandamide transporter, the inhibition of which leads to increased levels of endogenous cannabinoids (4, 24). As shown here, AM404 and structurally related fatty acid derivatives inhibit both COX-1 and COX-2 (Fig. 1). Thus, it is not surprising that AM404 has anti-nociceptive properties and potentiates the analgesic effect of anandamide in the mouse hot plate test (4, 6). Arachidonoyldopamine and oleyl vanillylamide (olvanil), other members of an increasing group of fatty acid amides acting on both vanilloid receptors and the endogenous cannabinoid system (24), have analgesic and anti-inflammatory effects and influence body temperature in a variety of *in vivo* assays (5, 16).

The endogenous fatty acid amide anandamide (arachidonoylethanolamide), which is an agonist at cannabinoid (22) and vanilloid (28) receptors, is hydrolysed to arachidonic acid and ethanolamine by a fatty acid amide hydrolase (FAAH) (9, 10). This enzyme may also act in the reverse direction, causing synthesis of anandamide from arachidonic acid and ethanolamine (10). The structures of acetaminophen and AM404 differ only with regard to the length of the hydrocarbon chain. We have shown that acetaminophen, following deacetylation to its primary amine *p*-aminophenol, is conjugated with arachidonic acid to form AM404 (Fig. 3, 4 and 5). Our discovery of AM404 as a metabolite of acetaminophen produced locally in the central nervous system provides an explanation for the mechanism of action of this widely consumed analgesic and antipyretic agent. Other primary amines, such as dopamine and serotonin, are also conjugated with arachidonic acid to form their respective arachidonoylderivatives (Fig. 6), indicating that this biochemical pathway represents a general mechanism for biotransformation of primary amines into fatty acid amides.

An important implication of our discovery is that substances having little or no activity in screening assays *in vitro* may undergo biotransformation *in vivo* to active fatty acid

derivatives. Fatty acid conjugation may, thus, be an important mechanism for bioactivation of low molecular weight compounds.

In a first aspect, the invention provides a method for screening for compounds or molecules that undergo conjugation with fatty acids in the mammal body, including humans. The method can further be used for testing whether a compound or molecule has the ability to undergo such a conjugation. In embodiment, tissue homogenate, preferably of liver or brain, or an enzyme, preferably an esterase or amidase, including fatty acid amide hydrolase (FAAH), or a mixture of enzymes is provided in a kit. The homogenate, enzyme or mixture of enzymes can be present in the kit as the sole component, or it can be present as part of a composition, alone or in combination with a fatty acid or a mixture of fatty acids, or other compounds, solutions or devices necessary or desirable for use of the kit.

Thus, the present invention relates to a method and a kit for carrying out the method, whereby the method for screening of compounds or molecules that undergo conjugation with fatty acids present in a mammal body, including humans, is characterized in that a compound to be tested is brought into contact with a fatty acid together with a tissue homogenate or an enzyme, whereupon any conjugation between the compound and any fatty acid present in said homogenate or enzyme solution is determined.

The determination is preferably made with regard to presence of fatty acid amides, esters or thioesters.

The enzyme is preferably an esterase or an amidase, including fatty acid amide hydrolase.

The tissue homogenate is preferably a homogenate of liver and/or brain.

The fatty acid amide, ester or thioester is preferably a derivative acting on the vanilloid receptor, the cyclo-oxygenases and/or the endocannabinoid system, including the cannabinoid receptors and the anandamide transporter.

The fatty acid has typically a chain length of between 12 and 24 carbon atoms.

The fatty acid is saturated or unsaturated, having between 1 and 5 double bonds.

Preferably, the fatty acid is a fatty acid present in the mammal body, including humans, whereby the fatty acid is preferably arachidonic acid, linolenic acid, linoleic acid, oleic acid, palmitoleic acid, lignoceric acid, behenic acid, arachidic acid, stearic acid, palmitic acid myristic acid and lauric acid.

The fatty acid can also be a product of the cyclo-oxygenase, lipoxygenase and cytochrome P450 monoxygenase pathways, including prostaglandins, thromboxanes, leukotriens, lipoxins, and hydroxy-, epoxy- or hydroperoxyderivatives of eicosatetraenoic and eicosatrienoic acids.

In another aspect, the invention provides a kit for conjugation of a compound or molecule with a fatty acid according to existing knowledge in the art of organic chemistry. The bond between the fatty acid and the compound or molecule can be an amide, ester or thioester bond. The synthesis of such fatty acid derivatives can be carried out following known procedures, such as those described in refs 8, 13, 17 and 25. In embodiment, the kit contains an enzyme, preferably FAAH, or a mixture of enzymes, a fatty acid or a mixture of fatty acids, or a combination of these. Alternatively, the kit can contain modified or activated fatty acids or enzymes, according to existing knowledge in the arts of organic and combinatorial chemistry, to provide optimal substrates or conditions for the conjugation reaction to occur.

The enzyme(s), fatty acid(s) or modified fatty acid(s) can be present in the kit as the sole component, or it can be present as part of a composition, alone or in combination with other compounds, enzymes, solutions or devices necessary or desirable for use of the kit. The enzyme(s), fatty acid(s) or modified fatty acid(s) can be present as dry substance, solution or attached to a suitable solid phase or polymer. For example, the fatty acid can be converted to a fatty acid chloride and anchored to a polymer to form an activated ester using known procedures, such as those described in refs 8 and 13. The fatty acid derivatives produced by the kit can subsequently be tested in screening assays to determine their biological activity.

The kit for conjugation of a compound or molecule with a fatty acid to yield a fatty acid amide, ester or thioester comprises a fatty acid together with at least one conjugation promoting substance, or a modified (activated) fatty acid.

The promoting substance can be a chemical reagent and/or an enzyme, preferably an esterase and/or an amidase, including fatty acid amide hydrolase.

The fatty acid or modified fatty acid has typically a chain length of between 12 and 24 carbon atoms.

The fatty acid or modified fatty acid is saturated or unsaturated, having between 1 and 5 double bonds.

Preferably, the fatty acid is a fatty acid present in the mammal body, including humans, whereby the fatty acid is preferably arachidonic acid, linolenic acid, linoleic acid, oleic acid, palmitoleic acid, lignoceric acid, behenic acid, arachidic acid, stearic acid, palmitic acid myristic acid and lauric acid.

The fatty acid can also be a product of the cyclo-oxygenase, lipoxygenase and cytochrome P450 monoxygenase pathways, including prostaglandins, thromboxanes, leukotriens, lipoxins, and hydroxy-, epoxy- or hydroperoxyderivatives of eicosatetraenoic and eicosatrienoic acids.

In the kit the enzyme, fatty acids or modified fatty acids are bound to a solid phase or present in a solution.

The fatty acid amide, ester or thioester is preferably a derivative acting on the vanilloid receptor, the cyclo-oxygenases and/or the endocannabinoid system, including the cannabinoid receptors and the anandamide transporter.

Legends to figures

Figure 1. a, No effect of acetaminophen (AcAP) and *p*-aminophenol (AP) on COX-1 and COX-2 activity in isolated enzyme preparations (n = 4-5). **b**, AM404 concentration-dependently inhibited both COX-1 and COX-2 activity. Indomethacin (10 μM) and the COX-2 selective inhibitor NS-398 (10 μM) almost abolished COX-1 ($6 \pm 0.4\%$, n = 4) and COX-2 ($11 \pm 2\%$, n = 6) activity, respectively (not shown). COX-1 and/or COX-2 were

also inhibited by the fatty acid derivatives arvanil (97% and 95%), arachidonoyldopamine (96% and 93%), arachidonoyl-3-methoxytyramine (94% and 77%), arachidonoylserotonin (71% and 2%), olvanil (97% and 93%), anandamide (14% and 44%) and 2-arachidonoylglycerol (11% and 67%), respectively (10 μM each; duplicate measurements). COX activity was measured as prostaglandin formation in the presence of 10 μM arachidonic acid.

Figure 2. Acetaminophen and *p*-aminophenol, in contrast to AM404, do not act on native vanilloid receptors in rat isolated mesenteric arteries. **a**, Representative traces showing no response to acetaminophen (AcAP) or *p*-aminophenol (AP) in arterial segments contracted with phenylephrine (n = 5). Capsaicin (CAP) always relaxed these arteries (pEC_{50} = 8.36 ± 0.05, n = 5). Dashed line indicates the basal tension level before addition of drugs. **b**, Concentration-response curves for capsaicin in arterial segments contracted with phenylephrine after treatment with 1 mM acetaminophen (triangles), 100 μM *p*-aminophenol (squares) or vehicle (circles) for 30 min (n = 5). **c**, AM404 is a potent vasodilator (open circles) of arterial segments contracted with phenylephrine (pEC_{50} = 7.80 ± 0.01, n = 11). The action of AM404 is inhibited by the competitive vanilloid receptor antagonist capsazepine (3 μM; filled circles; n = 5) and the non-competitive vanilloid receptor antagonist ruthenium red (1 μM; diamonds; n = 4). AM404 was unable to relax arteries pre-treated with capsaicin (1 μM) for 30 min (n = 4; not shown) to cause vanilloid receptor desensitisation and/or depletion of sensory neuropeptides (49). Broken line (triangles) shows the relaxant effect of "endogenous" AM404 from rat homogenates incubated with *p*-aminophenol (mean of 4 arterial segments from the same rat). "Endogenous" AM404 was purified using LC and quantified by LC/MS-MS as described. Tension traces show relaxant responses to increasing concentrations of exogenous (upper trace) and "endogenous" (lower trace) AM404.

Figure 3. Acetaminophen is metabolised to the primary amine *p*-aminophenol, which is further conjugated with arachidonic acid to form the bioactive fatty acid amide *N*-(4-hydroxyphenyl)-5,8,11,14-eicosatetraenamide (AM404).

Figure 4. Formation of AM404 and *p*-aminophenol in rat after intraperitoneal injection of acetaminophen (30 - 300 mg kg^{-1}) and its inhibition by PMSF (10 mg kg^{-1}). **a,b,**

Representative chromatograms of samples obtained from rat brains showing (a) the presence of AM404 (23.4 pmol g^{-1}) in an animal treated with acetaminophen and (b) no AM404 in an animal injected with vehicle. The tandem mass spectrometer was operated to select the protonated molecular ion of AM404 at *m/z* 396.1 in the first quadruple mass separator, while the mass fragment at 109.8 after fragmentation in the collision cell (corresponding to the protonated *p*-aminophenol fragment) was selected by the second quadruple. **c,d,** Identification of AM404 and *p*-aminophenol in various tissues obtained from rats after exposure to acetaminophen or vehicle for 20 min *in vivo* (n = 4 - 5; *P<0.016 compared to vehicle). **e,f,** Quantification of AM404 and *p*-aminophenol in brain after administration of different doses of acetaminophen (n = 6 – 10). **g,** PMSF abolishes the formation of AM404 (filled bars) but only partly inhibits the formation of *p*-aminophenol (non-filled bars) in brain after administration of acetaminophen (n = 5).

Figure 5. The formation of AM404 in rat brain homogenates is dependent on *p*-aminophenol and is sensitive to the enzyme inhibitor PMSF. **a**, *p*-Aminophenol (10 μM; circles), but neither acetaminophen (100 μM; triangles) nor vehicle (not shown), causes a production of AM404 in brain homogenates (n = 4). **b**, Formation of *p*-aminophenol from acetaminophen (100 μM) was detected in liver (circles), but not in brain (triangles) homogenates (n = 4). No *p*-aminophenol could be detected in homogenates incubated with vehicle (n = 4). **c, d**, Brain homogenates were incubated for 1 hour with either *p*-aminophenol plus arachidonic acid (each 100 μM) to generate AM404 or [$^2H_8$]-anandamide (10 μM) to study its hydrolysis. Pre-incubation for 1 hour with PMSF inhibits (**c**) AM404 production and (**d**) [$^2H_8$]-anandamide hydrolysis, measured as [$^2H_8$]-arachidonic acid formation (n = 4).

Figure 6. The formation of arachidonoyldopamine and arachidonoylserotonin in rat brain homogenates is sensitive to the enzyme inhibitor PMSF. Homogenates were incubated with arachidonic acid (AA; 100 μM) alone or combined with (**a**) dopamine (DA; 100 μM) or (**b**) serotonin (5-HT; 100 μM) for 1 hour (n = 3). The production of arachidonoyldopamine and arachidonoylserotonin was inhibited by PMSF (100 μM), but not by its ethanol vehicle (EtOH; 0.1%), added to homogenates 1 hour before the addition of arachidonic acid (in ethanol 0.1%) plus either dopamine or serotonin (n = 3).

Figure 7. Vanilloid receptor-dependent vasodilator action of different arachidonoyl derivatives in rat isolated mesenteric in arterial segments contracted with phenylephrine. Concentration-response curves for (**a**) 1-arachidonoylglycerol (1-AG) and (**b**) 2-arachidonoylglycerol (2-AG) in the absence (filled circles) and presence (filled triangles) of the competitive vanilloid receptor antagonist capsazepine (1 μM). The 3-methoxytyramine (circles), dopamine (triangles) and tyramine (squares) derivatives of arachidonic acid also induced concentration-dependent relaxation (**c**). None of the agonists elicited a relaxation after pre-treatment with 10 μM capsaicin for 30 min (open symbols) to cause vanilloid receptor desensitisation and/or depletion of sensory neuropeptides (49).

Experimental part

The invention will now be described in more detail with reference to specific examples of the invention, which are not intended to be, and should not be construed as, limiting the scope of the invention in any way.

Materials and methods

Synthesis. The compounds of the present invention were synthesised in accordance with common practise, whereby the starting materials were synthesised as well, or were bought in bulk from common suppliers of organic chemicals.

***In vivo* experiments.** Acetaminophen (30, 100 or 300 mg kg^{-1}) or vehicle (saline) at a volume of 2 - 3 ml was given to female Wistar-Hannover rats (200 - 300 g) by an intraperitoneal injection. Some rats were pretreated with PMSF (10 mg kg^{-1}) or vehicle (saline:PEG 6000; 1:10 w/w) given subcutaneously (2 - 3 ml) 20 min before administration of acetaminophen. Approximately 20 min after injection of acetaminophen, the animals were killed to collect brain, liver, spinal cord and arterial blood. The tissues were homogenised in a Tris buffer (10 mM, pH 7.6) containing EDTA (1 mM). PMSF (0.1 mM) and ascorbic acid (0.3 mM) were also present in the Tris buffer and added to the blood samples to prevent degradation of fatty acid amides and *p*-aminophenol, respectively. Aliquots (200 μl) of blood and homogenates were precipitated with 1 ml ice-cold acetone, containing 1 μM [$^2H_8$]-labelled anandamide as internal standard. The samples were kept on ice until the acetone phase was evaporated *in vacuo*.

Tissue homogenate experiments. The brain, liver, spinal cord and dorsal root ganglia from female Wistar-Hannover rats (250 g) were homogenised in a Tris buffer (10 mM, pH 7.6), containing EDTA (1 mM), to give 90 - 330 mg tissue ml^{-1}. We carried out experiments in aliquots of 200 µl homogenate at 37°C as further explained in the text. The reactions were stopped by adding 1 ml ice-cold acetone containing 1 µM [$^2H_8$]-anandamide. The samples were kept on ice until the acetone phase was evaporated in vacuo.

Quantitative analyses. The extraction residues were reconstituted in 100 µl methanol except for *p*-aminophenol, for which 100 µl 0.5% acetic acid was used. The quantitative analyses were performed using a Perkin Elmer 200 liquid chromatography system with autosampler (Applied Biosystems), coupled to an API 3000 tandem mass spectrometer (Applied Biosystems/MDS-SCIEX). All mobile phases were water-methanol gradients, containing 0.5% acetic acid, and the flow rate was 200 µl min^{-1} except for arachidonic acid where it was 400 µl min^{-1}.

AM404, arachidonoyldopamine, arachidonoylserotonin and anandamide. Sample aliquots of 5 µl were injected on a Genesis C_8 column (20 × 2.1 mm; Jones). Initially, the mobile flow was 25% water for 5.5 min. Then a linear gradient to 100% methanol was applied in 0.2 min and the mobile phase was kept at 100% methanol for 2.3 min, after which the column was reconditioned in 25% water for 2 min. The electrospray interface was operating in the positive ion mode at 370°C, the ion spray voltage was 4500 volts and the declustering potential was 40 volts. M/z 396.1/109.8 with a collision energy of 27 volts was used for the AM404 determinations. M/z 440.2/153.5 with a collision energy of 25 volts, m/z 463.2/159.6 with a collision energy of 39 volts and m/z 348.2/61.6 with a collision energy of 35 volts were used for arachidonoyldopamine, arachidonoylserotonin and native anandamide, respectively. M/z 356.4/62.2 with a collision energy of 35 volts was used for the internal standard [$^2H_8$]-labelled anandamide.

***p*-Aminophenol.** Sample aliquots of 2 µl were injected on a Genesis phenyl column (150 × 2.1 mm; Jones). The mobile flow was initially 97% water for 2 min. Then a linear gradient to 100% methanol was applied in 1 min and the mobile phase was kept at 100% methanol for 2 min, after which the column was reconditioned in 97% water for 3 min. The electrospray ion source was set at 450°C and used in the positive ion mode. The ion spray

voltage and declustering potential were set to 4500 volts and 55 volts, respectively. *M/z* 109.9/64.6 with a collision energy of 31 volts was used for the quantitative determinations.

[$^2H_8$]-Arachidonic acid. Sample aliquots of 5 µl were injected on a Genesis C_{18} column (50 × 2.1 mm; Jones). The HPLC was operated isocratically at 20% water and 80% methanol. The electrospray ion source was operating in the negative ion mode at 370°C, the ion spray voltage was -3000 volts and the declustering potential was -120 volts. *M/z* 310.8/267.0 with a collision energy of -22 volts was used for the quantitative determinations.

COX-1 and COX-2 assays. COX-1 and COX-2 activity was determined in the presence of 10 µM arachidonic acid using a COX (ovine) inhibitor screening assay (Cayman). Drugs were incubated with the enzyme preparation 8 min before application of arachidonic acid. Prostaglandin formation was used as a measure of COX activity and quantified via enzyme immunoassay (EIA).

Recording of tension. Experiments were performed on mesenteric arteries from female Wistar-Hannover rats (250 g) as described (49). Briefly, the arteries were cut into ring segments and mounted in tissue baths, containing aerated physiological salt solution (5% CO_2 and 95% O_2; 37°C; pH 7.4). Experiments carried out in the presence of N^G-nitro-L-arginine (0.3 mM) and indomethacin (10 µM) to eliminate any contribution of nitric oxide and cyclo-oxygenase products, respectively. We studied relaxant responses in preparations contracted with phenylephrine. When stable contractions were obtained, substances were added cumulatively to determine concentration-response relationships.

Calculations and statistics. Data are presented as means ± S.E.M. (vertical lines in figures), and *n* indicates the number of animals unless otherwise stated. GraphPad Prism 3.0 software was used for curve fitting (non-linear regressions) and calculations of pEC_{50} values. Mann-Whitney U-test or Student's t-test on log transformed values was used for statistical analysis. Statistical significance was accepted when $P < 0.05$.

Drugs. Acetaminophen, *p*-aminophenol, arachidonic acid, N^G-nitro-L-arginine, ascorbic acid, dopamine, phenylephrine, PMSF (phenyl-methyl-sulphonylfluoride), ruthenium red, serotonin (all from Sigma) and indomethacin (Confortid, Dumex) were dissolved in and

diluted with distilled water. AM404 (*N*-(4-hydroxyphenyl)-5,8,11,14-eicosatetraenamide), capsaicin, capsazepine (all from Tocris); 1-arachidonoylglycerol, 2-arachidonoylglycerol, [$^2H_8$]-anandamide, [$^2H_8$]-arachidonic acid, arachidonoylserotonin, NS-398 (all from Cayman); anandamide (Biomol); arachidonoyldopamine, arachidonoyl-3-methoxytyramine and arachidonoyltyramine (Syntelec) were all dissolved in and diluted with ethanol. DMSO substituted ethanol as a solvent in the COX assays. The batch of acetaminophen contained no or less than 0.001% (w/w) of p-aminophenol as determined by LC/MS-MS.

Example 1

Fatty acid amides or esters as vanilloid receptor agonists. The vasodilator effects of AM404 and other fatty acid derivatives, capsaicin, acetaminophen and *p*-aminophenol in isolated segments of rat mesenteric arteries, a well-defined and very sensitive bioassay system of vanilloid receptor active drugs (49), were examined to assess their vanilloid receptor agonistic activity. As shown in Figs 2 and 7, AM404, capsaicin, arachidonoyldopamine, arachidonoyl-3-methoxytyramine, arachidonoyltyramine, 1-arachidonoylglycerol and 2-arachidonoylglycerol are all agonists at vanilloid receptors on vasodilator sensory nerves. Acetaminophen and *p*-aminophenol (in the presence of ascorbic acid to prevent its decomposition) neither induced vasorelaxation per se nor inhibited the effect of capsaicin in this bioassay system, indicating lack of agonist and antagonist actions on vanilloid receptors (Fig. 2).

Example 2

Fatty acid amides or esters as COX inhibitors. The effects of AM404, arvanil, arachidonoyldopamine, arachidonoyl-3-methoxytyramine, arachidonoylserotonin, olvanil, anandamide and 2-arachidonoylglycerol on COX-1 and COX-2 activity in isolated enzyme preparations were examined to assess their COX inhibitory activity. As shown in Fig. 1, these fatty acid derivatives inhibited COX-1 and/or COX-2 to various extents.

Example 3

Fatty acid conjugation of acetaminophen *in vivo*. Since the structures of acetaminophen and AM404 differs only with regard to the length of the hydrocarbon chain, we hypothesised that acetaminophen, following deacetylation to its metabolite *p*-aminophenol (32), is conjugated with arachidonic acid to form AM404 (Fig. 3). To test this proposal, we measured the levels of AM404 and *p*-aminophenol in various tissues of rat 20 min after

intraperitoneal injection of acetaminophen at a commonly used dose (300 mg/kg), which produces a robust analgesic effect in rodents (18, 22, 46). In all five animals exposed to acetaminophen, substantial levels of AM404 were observed in brain (15 ± 1.6 pmol g^{-1}). AM404 could also be detected in the spinal cord in two out of five animals, but was absent in liver and blood (Fig. 4). *p*-Aminophenol was present in all tissues (Fig. 4), of which the liver contained the highest levels (31 ± 3.2 nmol g^{-1}). The formation of AM404 from acetaminophen was dose-dependent as shown in brain (Fig. 4). Pre-treatment with the FAAH inhibitor PMSF abolished the formation of AM404 in brain, while the *p*-aminophenol content was reduced by 48% (Fig. 4). AM404 and *p*-aminophenol could not be detected in vehicle-treated animals (n = 4), whereas the levels of anandamide in the same samples of brain and spinal cord were 10 ± 0.5 pmol g^{-1} and 7.0 ± 0.6 pmol g^{-1}, respectively (n = 4).

Example 4

Fatty acid conjugation of acetaminophen and *p*-aminophenol *in vitro*. To further characterise the formation of AM404 and *p*-aminophenol, homogenates of rat brain and liver were incubated with *p*-aminophenol and acetaminophen for various time periods. Exposure to *p*-aminophenol (10 μM) produced a time-dependent formation of AM404 in brain homogenates, whereas incubation with acetaminophen (100 μM) did not result in any detectable levels of AM404 (Fig. 5a). Likewise, *p*-aminophenol could not be measured in brain homogenates incubated with acetaminophen (Fig. 5b). However, we cannot exclude that small but relevant amounts of *p*-aminophenol is produced in brain, since significant amounts of AM404 (14 ± 2.6 pmol g^{-1}, n= 4) was measured in brain homogenates incubated with a ten times higher concentration of acetaminophen (1 mM). Indeed, this amount of AM404 would correspond to a *p*-aminophenol concentration below the detection limit of the assay. Substantial amounts of *p*-aminophenol could, however, be detected in liver homogenates incubated with acetaminophen (Fig. 5b).

Since primary sensory nerves of dorsal root ganglia and connecting neurones in the spinal cord are potential cellular targets for analgesic drugs acting, directly or indirectly, on vanilloid and cannabinoid receptors (2, 36, 38), it was considered of interest to see if AM404 could be formed in these tissues. Indeed, formation of AM404 was demonstrated in homogenates of rat spinal cord (24 ± 2.2 pmol g^{-1}, n = 4) and dorsal root ganglia (10 ± 1.8

pmol g^{-1}, n = 4) incubated with *p*-aminophenol (10 μM) for 1 hour. The level of AM404 was enhanced 6-fold when the homogenates were supplemented with arachidonic acid (100 μM) and the *p*-aminophenol concentration was increased 10 times (spinal cord: 161 ± 20 pmol g^{-1}, n = 4; dorsal root ganglia: 62 ± 1.5 pmol g^{-1}, duplicate measurements of pooled homogenates from four animals).

As further shown in rat brain homogenates, AM404 is formed via an enzyme-dependent process. First, AM404 could not be detected in homogenates boiled for 10 min before incubated with *p*-aminophenol (100 μM) and arachidonic acid (100 μM) for 1 hour (n = 4). Second, phenyl-methyl-sulphonylfluoride (PMSF), a broad-spectrum protease, esterase and amidase inhibitor (13), concentration-dependently inhibited the formation of AM404 with a pEC_{50} value of 5.41 ± 0.03 (n = 4; Fig. 5c). This compound also inhibited the hydrolysis of anandamide with a similar pEC_{50} value (5.28 ± 0.07, n = 4; Fig. 5d).

Example 5

Fatty acid conjugation of primary amines. We also tested whether the endogenous monoamines dopamine and serotonin could be converted to their respective arachidonoylderivatives. Incubation of brain homogenates with dopamine or serotonin led to the production of substantial amounts of arachidonoyldopamine and arachidonoylserotonin (Fig. 6). The enzyme inhibitor PMSF almost abolished the formation of these fatty acid amides (Fig. 6). Thus, not only *p*-aminophenol, but also endogenous monoamines are enzymatically conjugated with arachidonic acid to form bioactive fatty acid amides.

REFERENCES

1. Abbott, F. V., and K. G. C. Hellemans. Phenacetin, acetaminophen and dipyrone: analgesic and rewarding effects. *Behavioural Brain Research* 112: 177-186, 2000.
2. Ahluwalia, J., L. Urban, M. Capogna, S. Bevan, and I. Nagy. Cannabinoid 1 receptors are expressed in nociceptive primary sensory neurons. *Neuroscience* 100: 685-688, 2000.
3. Bannwarth, B., F. Demotes-Mainard, T. Schaeverbeke, L. Labat, and J. Dehais. Central analgesic effects of aspirin-like drugs. *Fundamentals in Clinical Pharmacology* 9: 1-7, 1995.
4. Beltramo, M., N. Stella, A. Calignano, S. Y. Lin, A. Makriyannis, and D. Piomelli. Functional role of high-affinity anandamide transport, as revealed by selective inhibition. *Science* 277: 1094-1097, 1997.
5. Bisogno, T., D. Melck, M. Y. Bobrov, N. M. Gretskaya, V. V. Bezuglow, L. D. Petrocellis, and V. D. Marzo. *N*-acyl-dopamines: novel synthetic CB_1 cannabinoid-receptor ligands and inhibitors of anandamide inactivation with cannabimimetic activity *in vitro* and *in vivo*. *Biochemical Journal* 351: 817-824, 2000.
6. Burstein, S. H., R. G. Rossetti, B. Yagen, and R. B. Zurier. Oxidative metabolism of anandamide. *Prostaglandins & other Lipid Mediators* 61: 29-41, 2000.
7. Clissold, S. P. Paracetamol and phenacetin. *Drugs* 32: 46-59, 1986.
8. Cohen, B. J., H. Karoly-Hafeli, and A. Patchornik. Active esters of polymer-bound 4-hydroxy-3-nitrobenzophenone as useful acylating reagents. Application to peptide synthesis. *Journal of Organic Chemistry* 49: 922-924, 1984.
9. Cravatt, B. F., D. K. Giang, S. P. Mayfield, D. L. Boger, R. A. Lerner, and N. B. Gilula. Molecular characterization of an enzyme that degrades neuromodulatory fatty-acid amides. *Nature* 384: 83-87, 1996.
10. Deutsch, D. G., and S. A. Chin. Enzymatic synthesis and degradation of anandamide, a cannabinoid receptor agonist. *Biochemical Pharmacology* 46: 791-796, 1993.
11. Ferreira, S. H., B. B. Lorenzetti, and F. M. Correa. Central and peripheral antialgesic action of aspirin-like drugs. *European Journal Pharmacology* 53: 39-48, 1978.
12. Flower, R. J., and J. R. Vane. Inhibition of prostaglandin synthetase in brain explains the anti-pyretic activity of paracetamol (4-acetamidophenol). *Nature* 240: 410-411, 1972.
13. Hinzen, B. *Combinatorial chemistry: A practical approach*, Bannwarth, W. and Felder, E. (eds), Wiley-VCH, Weinheim, pp 211-212, 2000.

14. Hunskaar, S., and K. Hole. The formalin test in mice: dissociation between inflammatory and non-inflammatory pain. *Pain* 30: 103-114, 1987.

15. Insel, P. A. Analgesic-antipyretic and antiinflammatory agents and drugs employed in the treatment of gout. In: *Goodman & Gilman's The Pharmacological Basis of Therapeutics* (Ninth Edition ed.), edited by J. G. Hardman, A. G. Gilman and L. E. Limbird The McGraw-Hill Companies, Inc, 1996, p. 617-657.

16. Janusz, J. M., B. L. Buckwalter, P. A. Young, T. R. LaHann, R. W. Farmer, G. B. Casting, M. E. Loomans, G. A. Kerckaert, C. S. Maddin, E. F. Berman, R. L. Bohne, T. L. Cupps, and J. R. Milstein. Vanilloids. 1. Analogs of capsaicin with antinociceptive and antiinflammatory activity. *Journal of Medical Chemistry* 36: 2595-2604, 1993.

17. Khanolkar, A. D., V. Abadji, S. Lin, W. A. Hill, G. Taha, K. Abouzid, Z. Meng, P. Fan, and A. Makriyannis. Head group analogs of arachidonylethanolamide, the endogenous cannabinoid ligand. *J Med Chem* 39: 4515-9., 1996.

18. McCormack, K. Non-steroidal anti-inflammatory drugs and spinal nociceptive processing. *Pain* 59: 9-43, 1994.

19. Newton, J. F., C. H. Kuo, M. W. Gemborys, G. H. Mudge, and J. B. Hook. Nephrotoxicity of p-aminophenol, a metabolite of acetaminophen, in the fischer 344 rat. *Toxicology and Applied Pharmacology* 65: 336-344, 1982.

20. Pelissier, T., A. Alloui, F. Caussade, C. Dubray, A. Cloarec, J. Lavarenne, and A. Eschalier. Paracetamol exerts a spinal antinociceptive effect involving an indirect interaction with 5-hydroxytryptamine$_3$ receptors: *in vivo* and *in vitro* evidence. *Journal of Pharmacology and Experimental Therapeutics* 278: 8-14, 1996.

21. Petrocellis, L. D., T. Bisogno, J. B. Davis, R. J. Pertwee, and V. D. Marzo. Overlap between the ligand recognition properties of the anandamide transporter and the VR1 vanilloid receptor: inhibitors of anandamide uptake with negligible capsaicin-like activity. *Federation of European Biochemical Societies Letters* 483: 52-56, 2000.

22. Piomelli, D., A. Giuffrida, A. Calignano, and F. R. d. Fonseca. The endocannabinoid system as a target for therapeutic drugs. *Trends in Pharmacological Sciences* 21: 218-224, 2000.

23. Szallasi, A., and P. M. Blumberg. Vanilloid (capsaicin) receptors and mechanisms. *Pharmacological reviews* 51: 159-211, 1999.

24. Szallasi, A., and V. D. Marzo. New perspectives on enigmatic vanilloid receptors. *Trends in Neurological Sciences* 23: 491-497, 2000.

25. Walpole, C. S., R. Wrigglesworth, S. Bevan, E. A. Campbell, A. Dray, I. F. James, K. J. Masdin, M. N. Perkins, and J. Winter. Analogues of capsaicin with agonist activity as novel analgesic agents; structure-activity studies. 2. The amide bond "B-region". *Journal of Medical Chemistry* 36: 2373-2380, 1993.

26. Vaz, Z. R., V. C. Filho, R. A. Yunes, and J. B. Calixto. Antinociceptive action of 2-(4-bromobenzoyl)-3-methyl-4,6-dimethoxy benzofuran, a novel xanthoxyline derivative on chemical and thermal models of nociception in mice. *Journal of Pharmacology and Experimental Therapeutics* 278: 304-312, 1996.

27. Zygmunt, P. M., C. Huai-Hu, P. Movahed, D. Julius, and E. D. Högestätt. The anandamide transport inhibitor AM404 activates vanilloid receptors. *European Journal of Pharmacology* 396: 39-42, 2000.

28. Zygmunt, P. M., J. Petersson, D. A. Andersson, H. Chuang, M. Sorgard, V. Di Marzo, D. Julius, and E. D. Hogestatt. Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide. *Nature* 400: 452-457, 1999.

CLAIMS

1. Method for screening of compounds or molecules that undergo conjugation with fatty acids present in a mammal body, including humans, characterized in that a compound to be tested is brought into contact with a fatty acid together with a tissue homogenate or an enzyme, whereupon any conjugation between the compound and any fatty acid present in said homogenate or enzyme solution is determined.

2. Method according to claim 1, wherein the determination is made with regard to presence of fatty acid amides, esters or thioesters.

3. Method according to claims 1-2, wherein the tissue homogenate is a homogenate of liver and/or brain.

4. Method according to claims 1-2, wherein the enzyme is an esterase and/or an amidase, including fatty acid amide hydrolase.

5. Method according to claims 1-4, wherein the fatty acid amide, ester or thioester is a derivative acting on the vanilloid receptor, the cyclo-oxygenases and/or the endocannabinoid system, including the cannabinoid receptors and the anandamide transporter.

6. Method according to one or more of claims 1-4, wherein the fatty acid is a fatty acid present in the mammal body, including humans.

7. Method according to claim 6, wherein the fatty acid has a chain length of between 12 and 24 carbon atoms.

8. Method according to claim 7, wherein the fatty acid is saturated or unsaturated, having between 1 and 5 double bonds.

9. Method according to claim 6, wherein the fatty acid is arachidonic acid, linolenic acid, linoleic acid, oleic acid, palmitoleic acid, lignoceric acid, behenic acid, arachidic acid, stearic acid, palmitic acid myristic acid and lauric acid.

10. Method according to claim 6, wherein the fatty acid is a product of the cyclo-oxygenase, lipoxygenase or cytochrome P450 monoxygenase pathways, including prostaglandins, thromboxanes, leukotriens, lipoxins, and hydroxy-, epoxy- or hydroperoxyderivatives of eicosatetraenoic and eicosatrienoic acids.

11. Kit for conjugation of a compound or molecule with a fatty acid to yield a fatty acid derivative, comprising a fatty acid together with a conjugation promoting substance, or a modified (activated) fatty acid.

12. Kit according to claim 11, wherein the conjugation promoting substance is at least one enzyme.

13. Kit according to claim 12, wherein the enzyme is an esterase and/or an amidase, including fatty acid amide hydrolase.

14. Kit according to claim 11, wherein the fatty acid derivative is a derivative acting on the vanilloid receptor, the cyclo-oxygenases and/or the endocannabinoid system, including the cannabinoid receptors and the anandamide transporter.

15. Kit according to one or more of claims 11-13, wherein the fatty acid has a chain length of between 12 and 24 carbon atoms.

16. Kit according to claim 15, wherein the fatty acid is saturated or unsaturated, having between 1 and 5 double bonds.

17. Kit according to claims 15-16, wherein the fatty acid is a fatty acid present in the mammal body, including humans.

18. Kit according claim 17, wherein the fatty acid is arachidonic acid, linolenic acid, linoleic acid, oleic acid, palmitoleic acid, lignoceric acid, behenic acid, arachidic acid, stearic acid, palmitic acid myristic acid and lauric acid.

19. Kit according to claim 17, wherein the fatty acid is a product of the cyclo-oxygenase, lipoxygenase and cytochrome P450 monoxygenase pathways, including prostaglandins,

thromboxanes, leukotriens, lipoxins, and hydroxy-, epoxy- or hydroperoxyderivatives of eicosatetraenoic and eicosatrienoic acids.

20. Kit according to one or more of claims 11-19, wherein the enzyme, fatty acid or modified fatty acid is bound to a solid phase or present in a solution.

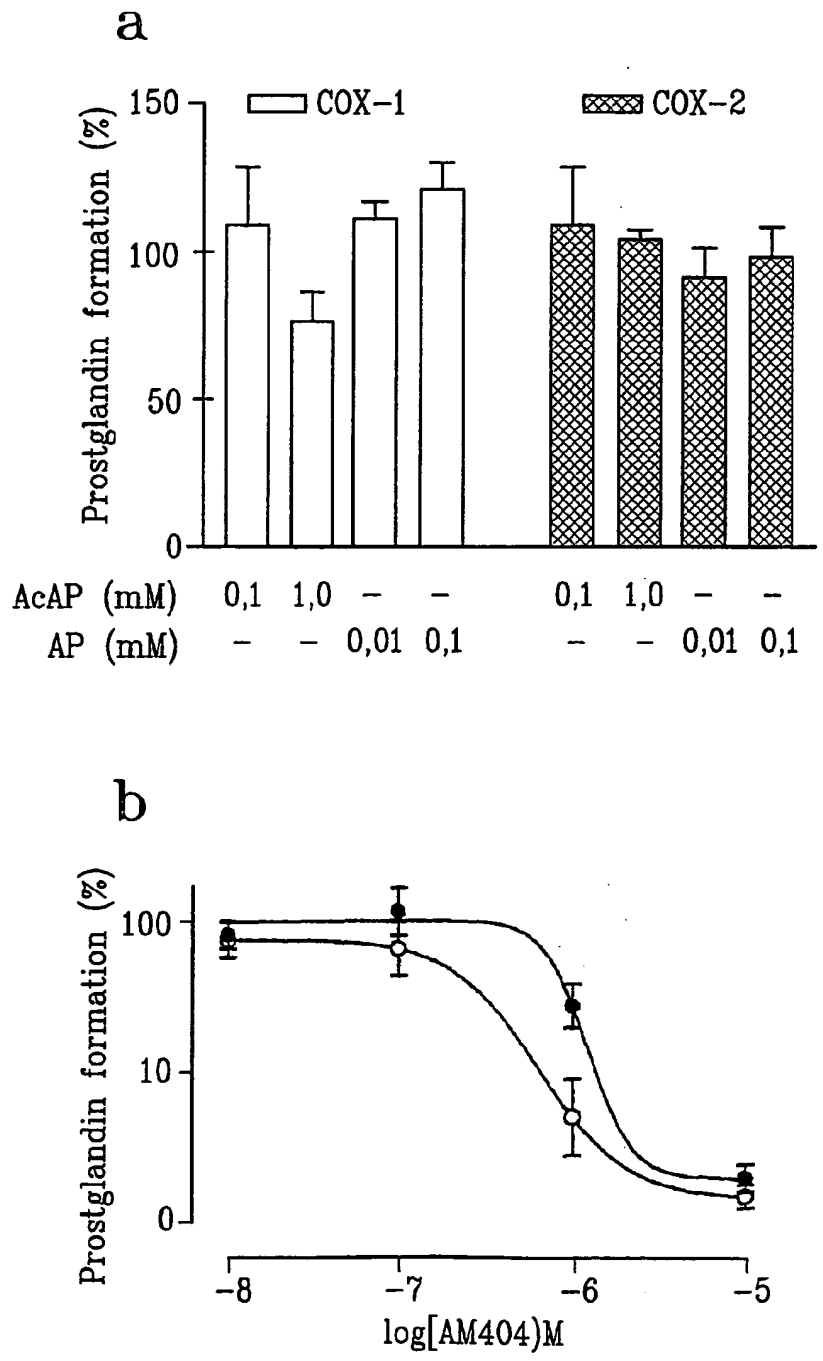

a
COX-1
COX-2
Prostglandin formation (%)
150
100
50
0
AcAP (mM) 0,1 1,0 – – 0,1 1,0 – –
AP (mM) – – 0,01 0,1 – – 0,01 0,1
b
Prostglandin formation (%)
100
10
0
–8
–7
–6
–5
log[AM404)M

FIG.1

a

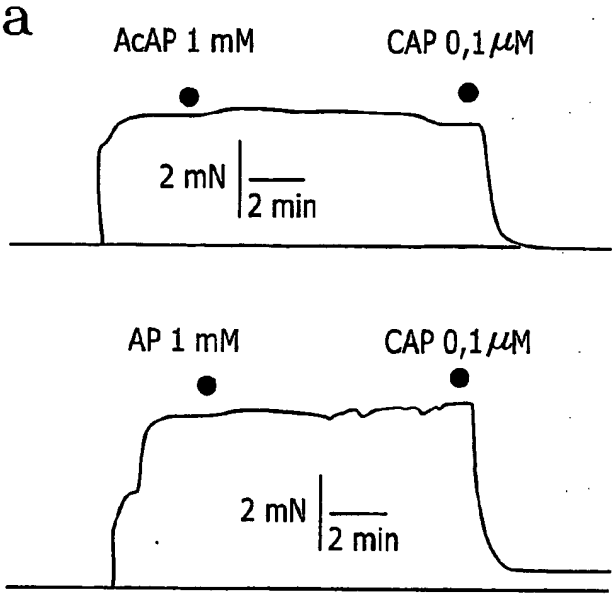


FIG.2a

b

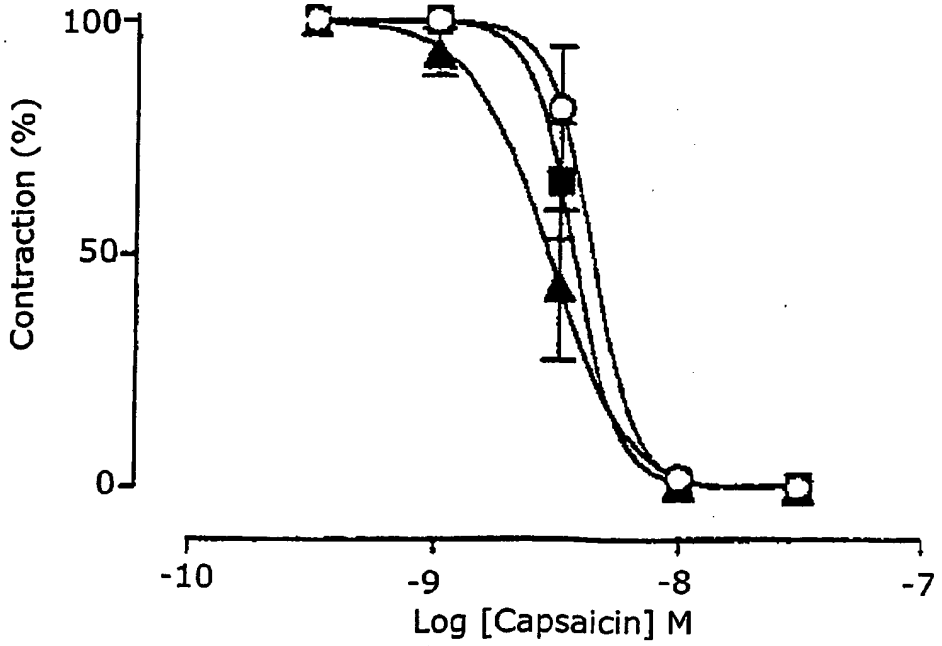


FIG.2b

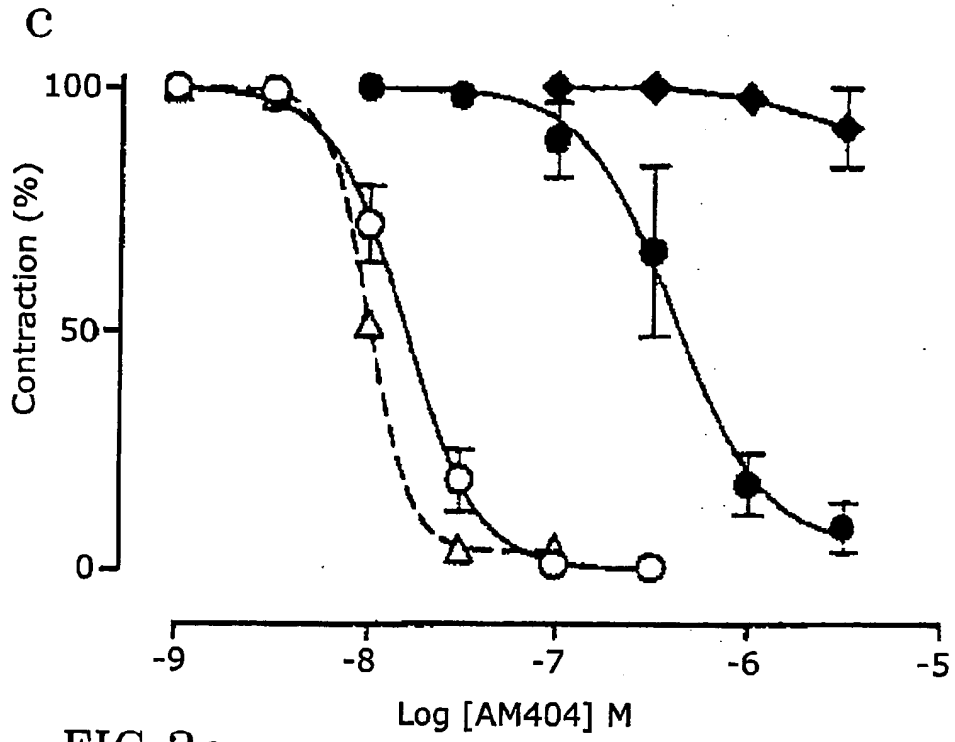

C
100
50
0
Contraction (%)
-9
-8
-7
-6
-5
Log [AM404] M

FIG.2c

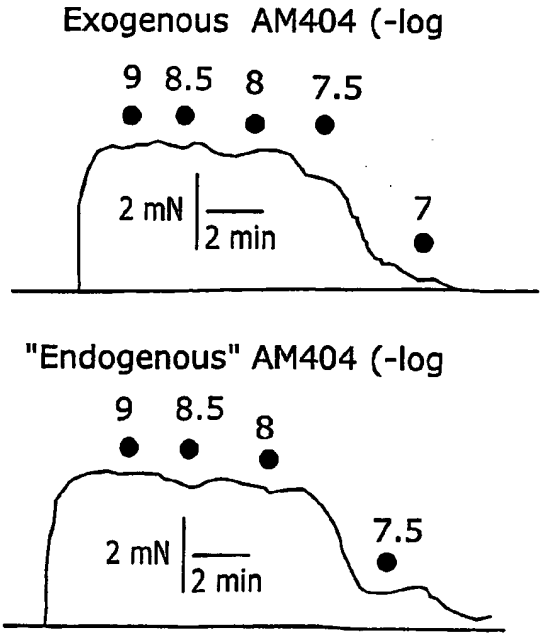
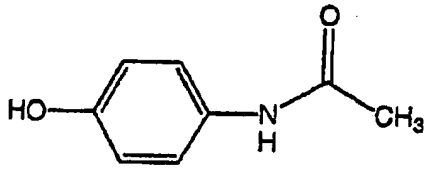
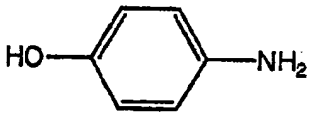

Exogenous AM404 (-log
9
8.5
8
7.5
2 mN
2 min
7
"Endogenous" AM404 (-log
9
8.5
8
2 mN
2 min
7.5

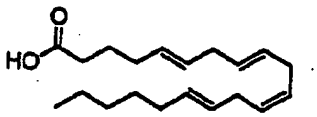
FIG.2d

Acetaminophen



p-Aminophenol





Arachidonic acid

AM 404

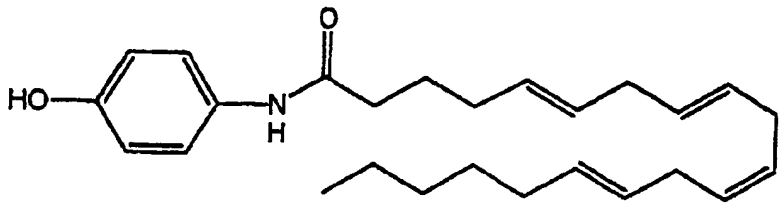


FIG.3

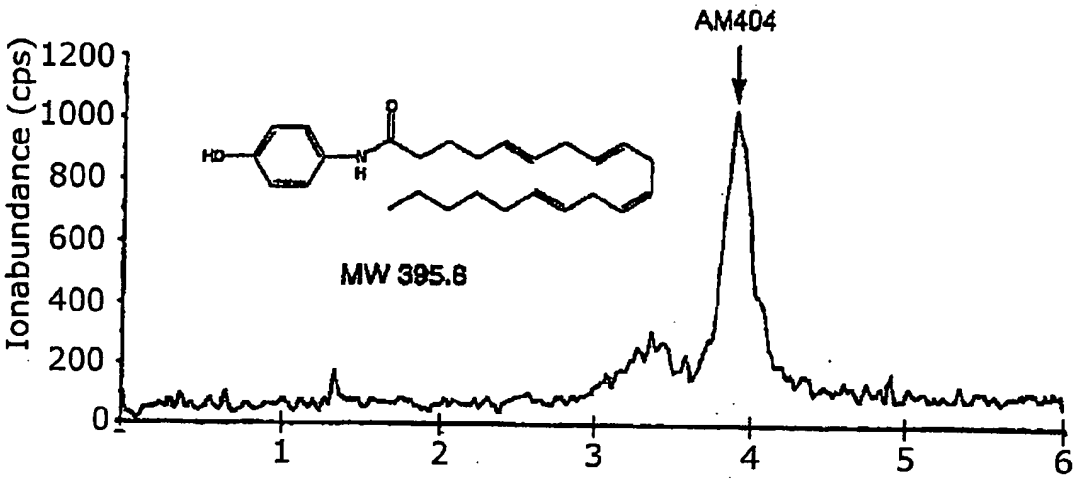


FIG.4a

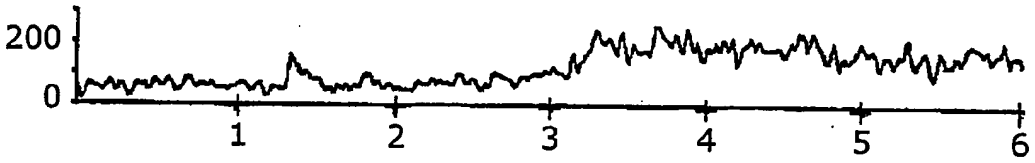


FIG.4b

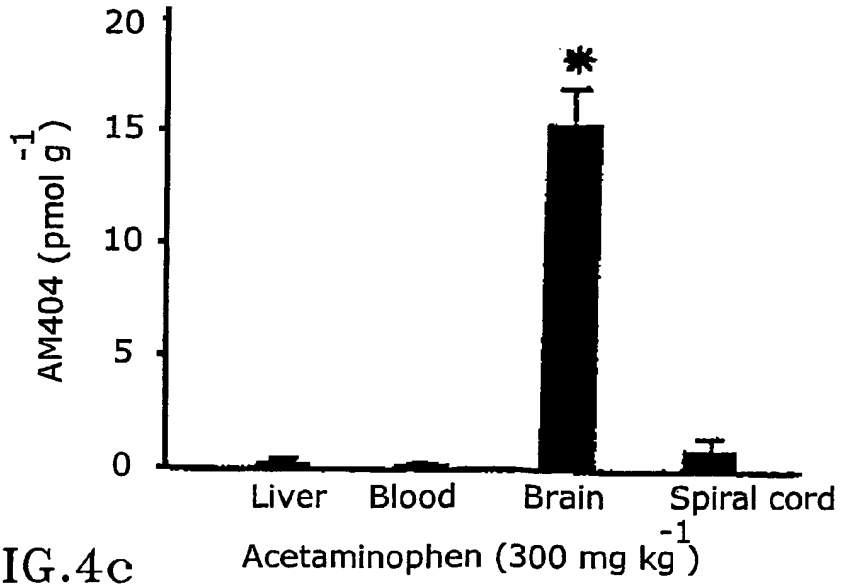


FIG.4c

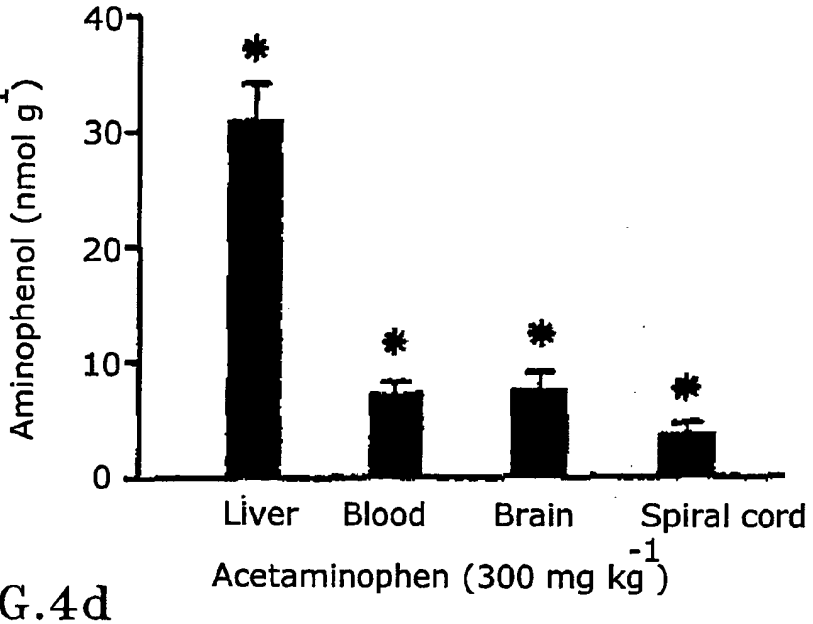

40
30
20
10
0
Aminophenol (nmol g-1)
Liver
Blood
Brain
Spiral cord
Acetaminophen (300 mg kg-1)

FIG.4d

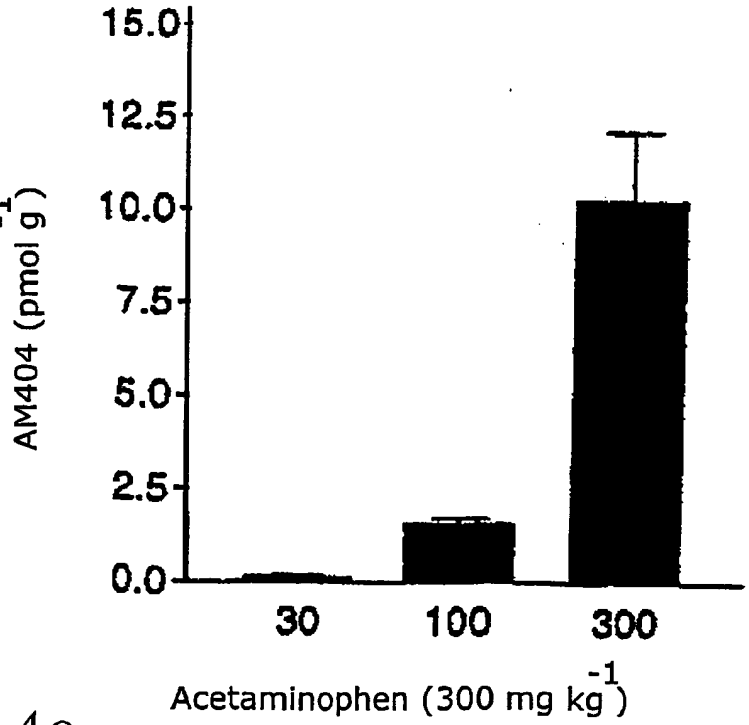

15.0
12.5
10.0
7.5
5.0
2.5
0.0
AM404 (pmol g-1)
30
100
300
Acetaminophen (300 mg kg-1)

FIG.4e

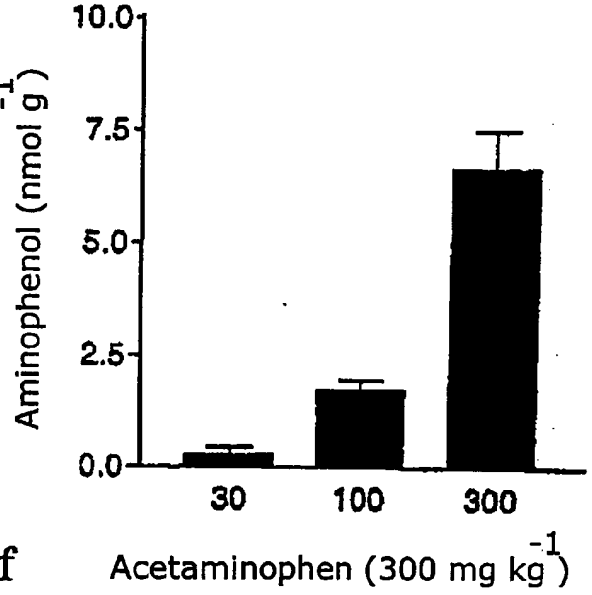

10.0
7.5
5.0
2.5
0.0
Aminophenol (nmol g-1)
30
100
300
Acetaminophen (300 mg kg-1)

FIG.4f

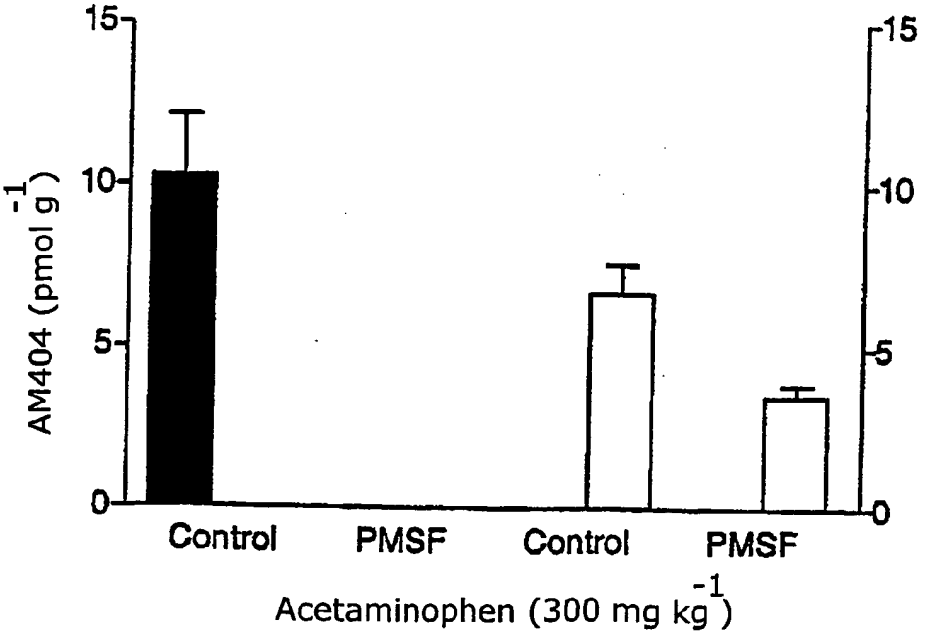

15
10
5
0
AM404 (pmol g-1)
Control
PMSF
Control
PMSF
Acetaminophen (300 mg kg-1)

FIG.4g

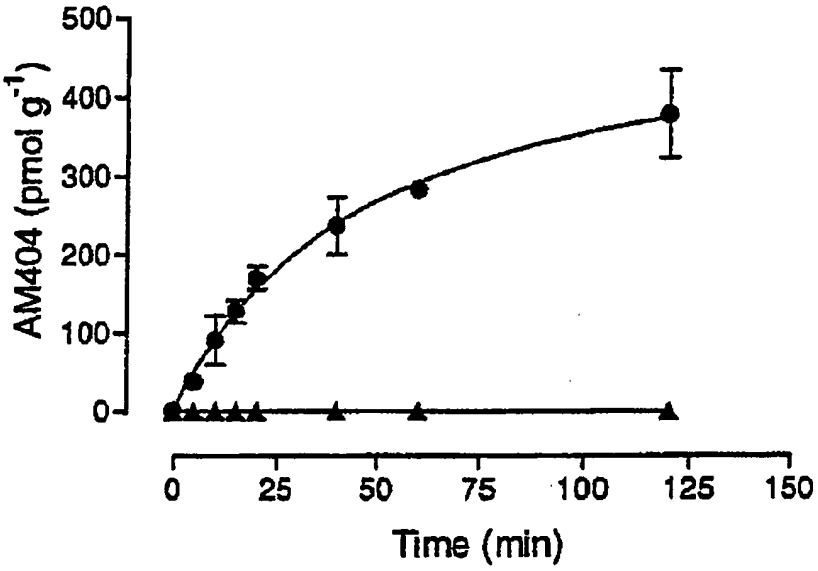

AM404 (pmol g-1)
500
400
300
200
100
0
0
25
50
75
100
125
150
Time (min)

FIG.5a

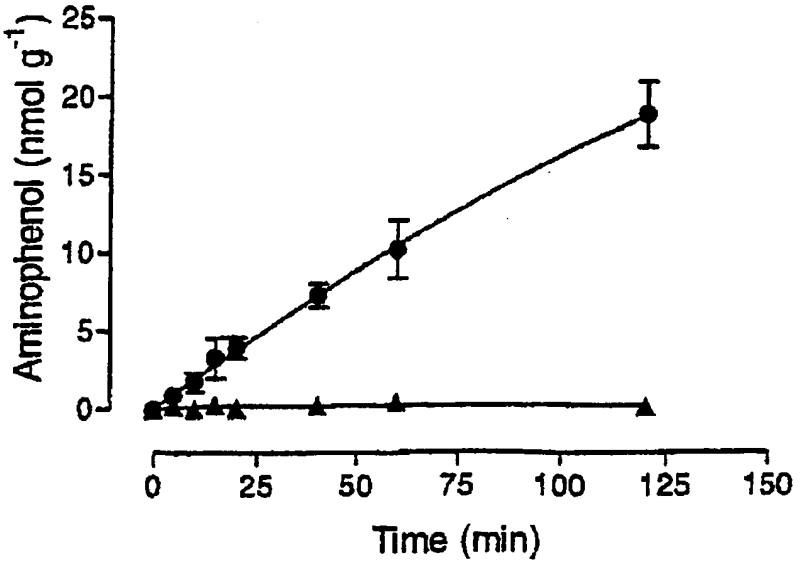

Aminophenol (nmol g-1)
25
20
15
10
5
0
0
25
50
75
100
125
150
Time (min)

FIG.5b

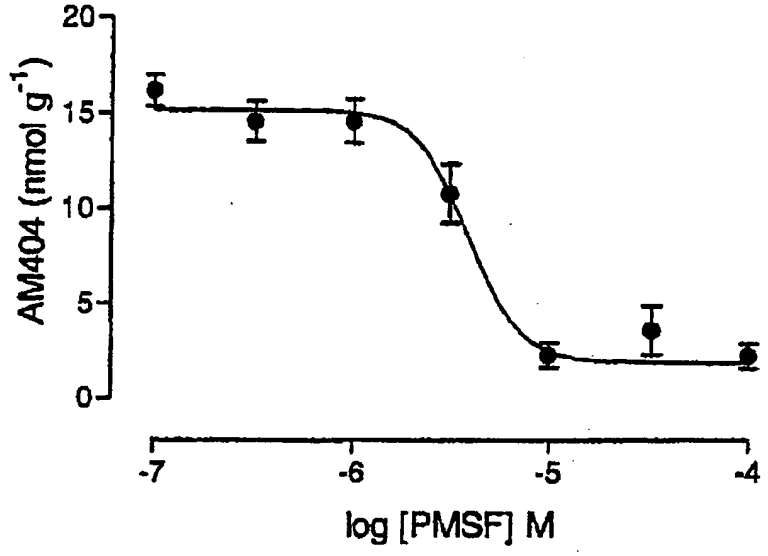

AM404 (nmol g-1)
20
15
10
5
0
-7
-6
-5
-4
log [PMSF] M

FIG.5c

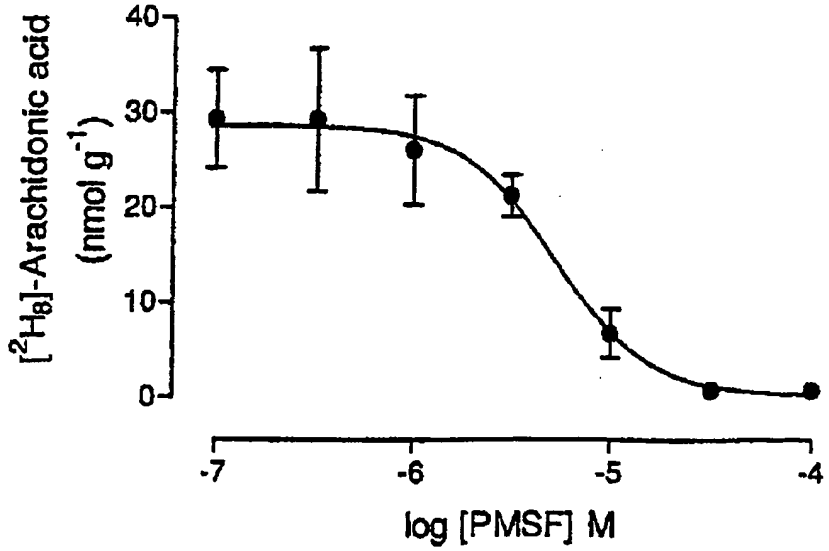

[2H8]-Arachidonic acid (nmol g-1)
40
30
20
10
0
-7
-6
-5
-4
log [PMSF] M

FIG.5d

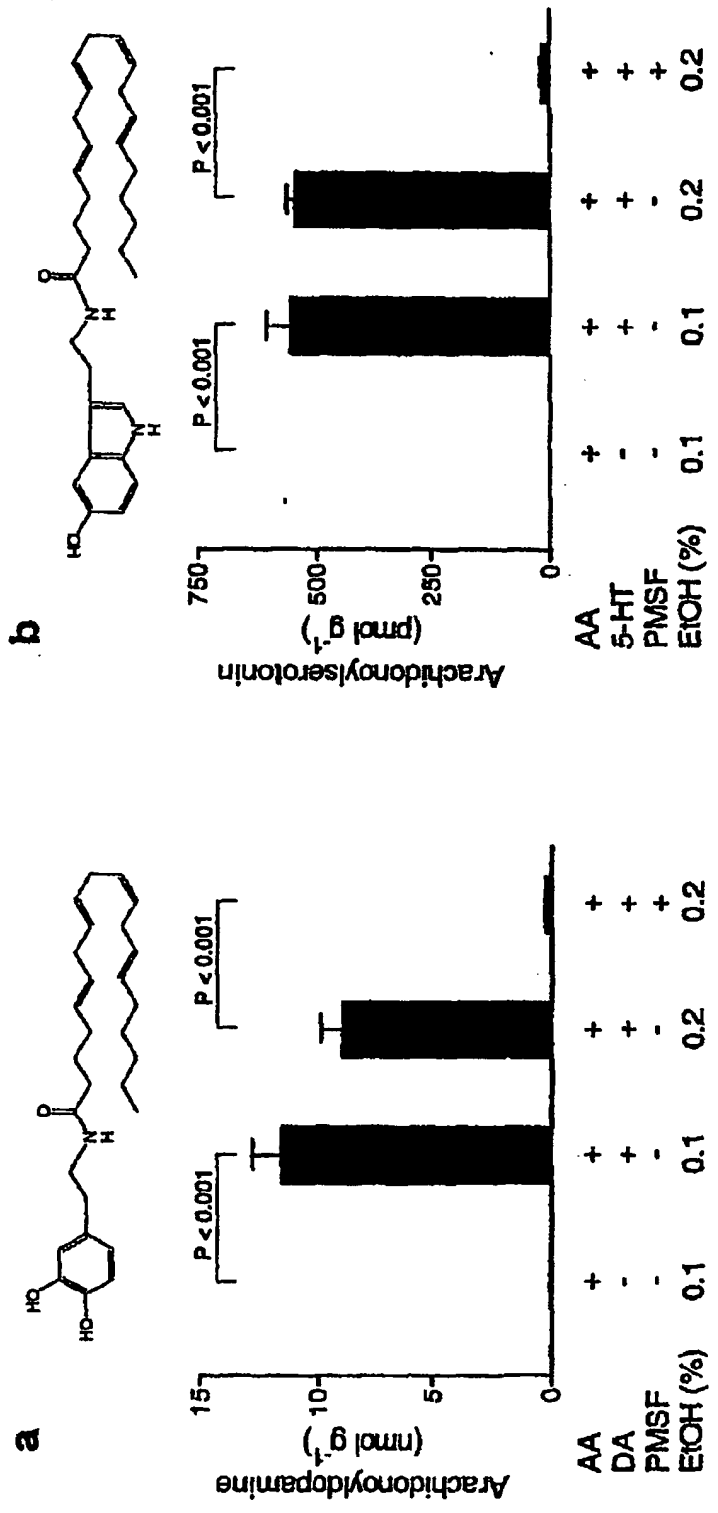

a
Arachidonyldopamine
(nmol g-1)
15
10
5
0
P < 0.001
P < 0.001
AA
DA
PMSF
EtOH (%)
+ - - 0.1
+ + - 0.1
+ + - 0.2
+ + + 0.2
b
Arachidonylserotonin
(pmol g-1)
750
500
250
0
P < 0.001
P < 0.001
AA
5-HT
PMSF
EtOH (%)
+ - - 0.1
+ + - 0.1
+ + - 0.2
+ + + 0.2

FIG.6

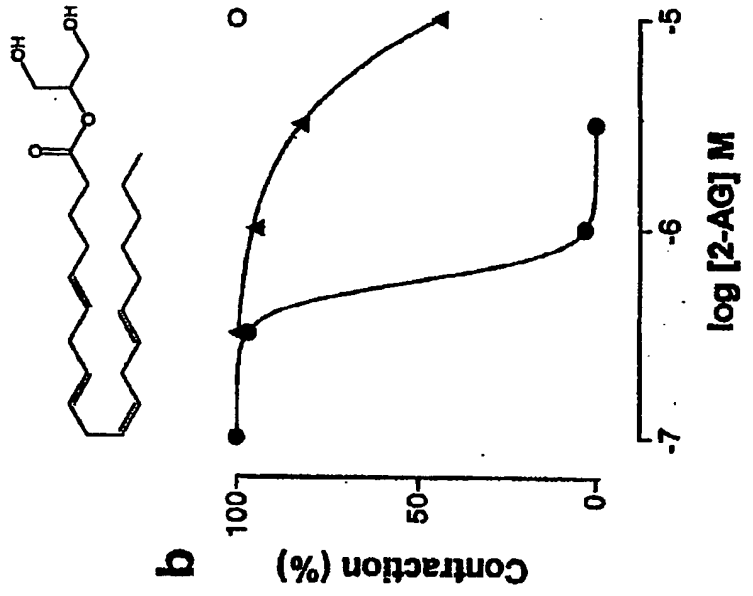

b
Contraction (%)
100
50
0
log [2-AG] M
-5
-6
-7
OH
OH
O
O

FIG. 7b

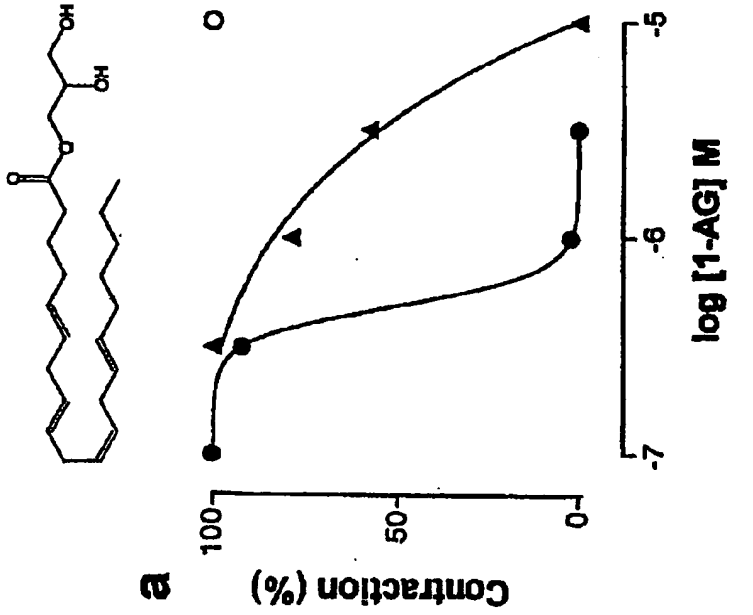

a
Contraction (%)
100
50
0
log [1-AG] M
-5
-6
-7
OH
OH
O
O

FIG. 7a

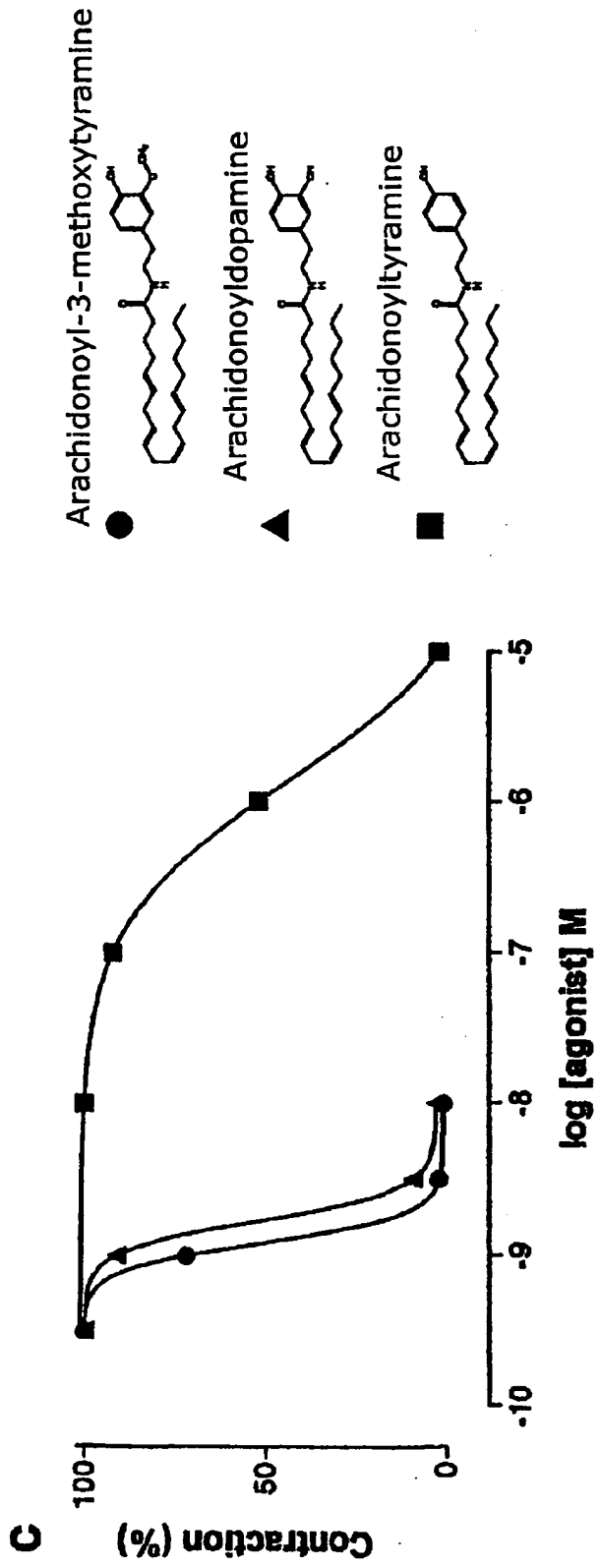

Arachidonoyl-3-methoxytyramine
Arachidonoyldopamine
Arachidonoyltyramine
C
Contraction (%)
100
50
0
-10
-9
-8
-7
-6
-5
log [agonist] M

FIG. 7c

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 02/01391

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: C12Q 1/00
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-INTERNAL, WPI-DATA, PAJ, BIOSIS, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Journal of Chromatography B, Volume 705, 1998, Bhupendra S. et al: "Single step thin-layer chromatographic method for quantitation of enzymatic formation of fatty acid anilides", page 269 - page 275, page 271, left column	1-10
X	--	11-20
A	Biochemical and Biophysical Research Communications, Volume 248, no RC988874, 1998, T. Bisogno et al: "Arachidonoylserotonin and Other Novel Inhibitors of Fatty Acid Amide Hydrolase", page 515 - page 522	1-10
X	--	11,14-20

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 November 2002	07 -11- 2002
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Anna Björklund/EÖ Telephone No. +46 8 782 25 00

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 02/01391

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Biochem. J., Volume 351, 2000, Tiziana Bisogno et al: "N-acyl-dopamines: novel synthedtic CB1 cannabinoid-receptor ligands and inhibitors of anandamide inactivation with cannabimimetic activity in vitro and in vivo", page 817 - page 824	1-10
X	--	11,14-20
A	WO 9412466 A1 (YISSUM RESEARCH DEVELOPMENT COMPANY OF THE HEBREW UNIVERSITY OF JERUSALEM), 9 June 1994 (09.06.94)	1-10
X	--	11,14-20

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE02/01391

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 1,9 and 10(partly)
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

see next sheet

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Present claims 1, 9 and 10 relate to an extremely large number of possible compounds. Support within the meaning of Article 6 PCT and / or disclosure within the meaning of Article 5 PCT is to be found, however, for only a very small proportion of the compounds claimed. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible.
Consequently, the search has been carried out for those parts of the claims that appear to be supported and disclosed, namely those parts related to the use of arachidonic acid according to the claimed method. The general aspect of using fatty acids according to the method has also been included in the search.

The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established will not be the subject of an international preliminary examination (Rule 66.1(e) PCT). This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

30/09/02

International application No.
PCT/SE 02/01391

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9412466 A1	09/06/94	AU 5733494 A	22/06/94
		CN 1097735 A	25/01/95
		CZ 9501361 A	15/11/95
		DE 69328492 D,T	07/09/00
		EP 0670826 A,B	13/09/95
		SE 0670826 T3	
		ES 2145118 T	01/07/00
		HU 73177 A	28/06/96
		HU 9501561 D	00/00/00
		IL 103932 A	18/02/97
		JP 8504195 T	07/05/96
		PL 309051 A	18/09/95
		SK 70895 A	13/09/95
		US 5618955 A	08/04/97
		ZA 9308947 A	02/08/94